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COMPLETE SPECIFICATION.

Improved Process of Purifying Plasminogen.

We, AMERICAN CYANAMID COMPANY, a Corporation organised under the laws of the State of Maine, United States of America, of 30 Rockefeller Plaza, New York, State of New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to an improved process for purifying plasminogen.

Human plasminogen has important uses in therapy. However, problems have been presented along two lines. In the first place, if plasminogen is to be administered, the higher the concentration the better as this reduces the volume which is needed, for example, in the case of injections, and this applies either where the plasminogen is administered as such or activated to plasmin by a suitable activator. The second problem arises due to the fact that sterility is necessary but it is extremely difficult to sterilize a relatively impure product which is not readily soluble.

In the past, the best method of obtaining a plasminogen concentrate is that described by Kline, J. Biol Chem., Volume 204, beginning at page 989. This is the purest plasminogen product which has been obtained hereto and its physical characteristics are described in an article by Shulman, J. Biol Chem., Volume 233, beginning at page 91. For simplicity throughout the Specification, this product will be referred to as a Kline plasminogen product and the method of producing the product as a Kline method.

In accordance with the invention, there is provided a process for purifying plasminogen, which comprises treating Kline plasminogen product with an aqueous solution of an amino compound which is lysine, epsilon amino caproic acid, arginine, tosyl arginine, histamine or a histidine compound, or a water-soluble salt or a water-soluble ester of any of these amino compounds, filtering and concentrating the filtrate, or alternatively adsorbing Kline plasminogen product on carboxymethyl cellulose and eluting. A much purer product of higher potency and higher activity is obtained. As the higher potency and higher activity results from the removal of contaminating impurities such as other proteins, the product is also more satisfactory as there is less danger of adverse reactions from foreign proteins. The amino compounds react or associate themselves in some way with the plasminogen rendering it soluble without solubilizing large amounts of other proteins. In carrying out the invention with carboxymethyl cellulose in ion-exchange columns the Kline plasminogen product may be adsorbed thereon preferably at a *p*H of 2.5 to 3.5 Elution may be effected with an aqueous acid producing a decreasing or increasing *p*H gradient, smooth or stepped. Salt gradient elution may also be used. The second method may be either continuous or in batch form. The solubilized product can be sterile, filtered with or without activation to plasmin at near physiological *p*H, and is satisfactory for human parenteral use. At the same time the potency is very markedly increased and so smaller amounts of the more concentrated product may be employed.

In the case of purification by lysine or lysine ester salts or the other amino compounds referred to above, to produce an aqueous solution of 75% or higher plasminogen concentration, the range of amino compound is from 0.05 to 0.18 mole.

[*Price 4s. 6d.*]

The invention will be illustrated in the following specific examples in which the parts are by weight unless otherwise specified.

EXAMPLE 1.

An aqueous solution of a Kline plasminogen product which had a *p*H of 8—10 was adjusted to the point of least solubility at *p*H 5.5 and stirred with various concentrations of lysine hydrochloride. Most of the plasminogen dissolved while much of the impurities remain undissolved, the undissolved proteins were removed by filtration and the supernatant fluid containing plasminogen concentrated to a white powder by freeze drying. The purity, that is to say units of plasminogen per milligram of nitrogen, and per cent solubility was determined. It will be seen that for a purity of 40,000 units per mg. lysine concentrations between 0.05 and 0.18 were suitable. Maximum purity and solubility are obtained between concentrations of 0.08 and 0.14.

EXAMPLE 2.

A procedure of Example 1 was repeated by dissolving one milligram of plasminogen at *p*H 2.5 adjusted to *p*H 10 and in the presence of 0.1 mg. lysine hydrochloride the *p*H adjusted to 5.5, stirred at room temperature and the precipitated impurity centrifuged out. The procedure was then repeated with two other samples of Kline product of somewhat higher purity. The results appear in the following table:—

TABLE.

Purity units/mg. Kline	Lysine treated	Recovery %
23,000	60,000	81
27,000	40,000	97

EXAMPLE 3.

Procedure of Example 2 is repeated replacing the lysine hydrochloride with other amino acid compounds. Control was checked for plasminogen solubility and showed less than 16%. Solubilities appear in the following table:—

TABLE.

Compound (0.05 M)	% Plasminogen in Solution
L-lysine.HCl	70
L-lysine ethyl ester.2HCl ...	82
epsilon-amino caproic acid ...	68
Tosyl L-arginine methyl ester.HCl	64
L-arginine methyl ester.HCl ...	60
histamine.2HCl	50
L-histidine.HCl	44
L-arginine.HCl	35

EXAMPLE 4.

Ten grams of carboxymethyl cellulose were washed several times in 1 M hydrochloric acid followed by water washing, the *p*H was then adjusted to 2.75 with 0.01 M sodium formate, buffer adjustment being with formic acid. A thin slurry of the carboxymethyl cellulose was then added to a chromatographic column under 10 pounds pressure to a volume of 28 milliliters (column measures 0.92 × 43 cm.). 675 optical density (D_{280}) units of Kline plasminogen product equilibrated by dialysis in the same buffer, was then added to the top of the column and allowed to adsorb. A gradient elution system was set up in which 0.1 M hydrochloric acid was gradually added with mixing to a formate buffer at *p*H 2.75 producing a gradually decreasing *p*H gradient. About one-half of the total protein containing no more than 5% of the plasminogen flowed through the column immediately. Plasminogen was then eluted at approximately *p*H 2.2 and the fractions pooled and determined by assay and protein determination. The purity and recovery are as follows: Kline starting material, 36,000 units/mg. N; chromatographed plasminogen, 86,000 units/mg. N; Recovery, 82%.

EXAMPLE 5.

The procedure of Example 4 was repeated but instead of a decreasing *p*H elution an increasing *p*H elution was obtained by using 0.01 molar sodium formate buffer at *p*H 3 to equilibrate the carboxymethyl cellulose and a gradient elution system prepared by mixing 0.25 molar sodium citrate, 0.1 molar lysine buffer at *p*H 6.5 with the formate buffer. This produced an elution with increasing *p*H and gave the following results: Kline starting material, 63,000 units/mg. N; chromatographed plasminogen, 102,600 units/mg. N; Recovery, 41.7%.

EXAMPLE 6.

The elution was varied by making a batchwise adsorption as follows: Kline plasminogen product was diluted at *p*H 2.8 using 0.05 M sodium formate buffer, to approximately 0.2 mg. N/ml. Ten grams of washed carboxymethyl cellulose were added to 100 ml. of the diluted plasminogen and mixed slowly for one hour at room temperature. Filtration with suction was then effected and the carboxymethyl cellulose resuspended in the same amount of buffer, stirred for one-half hour and refiltered. The carboxymethyl cellulose cake was then resuspended in buffer and adjusted to *p*H 2 with normal hydrochloric acid, mixed for an hour and filtered. A second elution at *p*H 2 with normal hydrochloric acid was then effected by mixing the cake therewith. The two eluates were combined, concen-

trated by dialysis and dried. The purity and recovery is as follows: Kline starting material, 52,000 units/mg. N; batch adsorbed and eluted, 75,000 units/mg. N; Recovery, 58%.

WHAT WE CLAIM IS:—

1. A process for purifying plasminogen, which comprises treating Kline plasminogen product with an aqueous solution of an amino compound which is lysine, epsilon amino caproic acid, arginine, tosyl arginine, histamine, or a histidine compound, or a water-soluble salt or a water-soluble ester of any of these amino compounds, filtering and concentrating the filtrate, or alternatively adsorbing Kline plasminogen product on carboxymethyl cellulose and eluting.

2. A process according to Claim 1, in which the Kline plasminogen product is reacted with a water solution of 0.05 to 0.18 molar concentration of said amino acid compound.

3. A process according to Claim 1, in which the Kline plasminogen product is adsorbed on carboxymethyl cellulose at a *p*H of 2.5 to 3.5.

4. A process according to Claim 3, in which the Kline plasminogen product is eluted with an aqueous acid producing an increasing or decreasing *p*H gradient.

5. A process of purifying plasminogen as claimed in Claim 1 and substantially as hereinbefore described.

6. Purified plasminogen whenever obtained by the process according to any one of the preceding claims.

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